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(21) International Application Number: PCT/EP97/03359 (22) International Filing Date: 26 June 1997 (26.06.97) (30) Priority Data: F196A000155 27 June 1996 (27.06.96) IT (71) Applicant (for all designated States except US): MINISTERO UNIVERSITA' RICERCA SCIENTIFICA E TECNOLOGICA [IT/IT]; Piazzale Kennedy, 20, I-00144 Rome (IT). (72) Inventors; and (75) Inventors/Applicants (for US only): MELE, Antonio [IT/IT]; Via E. Magnani, 16, I-51016 Montecatini Terme (IT). DE SANTIS, Rita [IT/IT]; Via Catilina, 14, I-00040 Pomezia (IT). PARENTE, Dino [IT/IT]; Via Plinio, 18, I-00040 Pomezia (IT). COLNAGHI, Maria, Ines [IT/IT]; Strada al Lago, 12, I-20090 Milan San Felice (IT). (74) Agent: GERVASI, Gemma; Notarbartolo & Gervasi, Corso di Porta Vittoria, 9, I-20122 Milan (IT).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: RECOMBINANT RIBOSOMAL INHIBITOR PROTEIN (RIP) AND USE AS IMMUNOCONJUGATE (57) Abstract <p>The following description refers to a new RIP protein (SEQ ID No: 3) the cDNA sequence expressing same (SEQ ID No: 2), its preparation and use in the preparation of chemical and recombinant conjugates having anticancer properties.</p>		

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RECOMBINANT RIBOSOMAL INHIBITOR PROTEIN (RIP) AND USE AS IMMUNOCONJUGATE**Field of the invention**

- 5 The present invention relates to a new protein which - after being transferred into the cell by a suitable vector - is capable of inhibiting the activity of ribosomes. Therefore, it can be used as an anticancer and/or antiviral agent.

State of the art

- 10 As known, proteins extracted from filamentous fungi of the genus *Aspergillus*, such as α -sarcin, restrictocin and mitogillin, can inhibit protein synthesis by inactivating eukaryotic ribosomes. The nucleotide sequence of genomic DNA expressing said proteins is also known.

- The antiviral activity of RIPs is related to the higher membrane permeability to the RIPs of virus-infected cells, with consequent injury to their ribosomes and consequent death of the infected cell. It follows that viral replication is interrupted.
15 It has recently been disclosed that several RIPs inhibit HIV replication and that a RIP preparation, thrichosantin (a protein extracted from the roots of *Trichosantes kirilowii*) was used in phase I/II clinical studies [Byers, VS. *et al.*, A phase I/II study of thrichosantin treatment of HIV diseases, *AIDS*, 4, 1189-1196 (1990)].

- 20 With a view to obtain selectively cytotoxic molecules, several of the known RIPs were bound to proteic and non-proteic vectors capable of transferring them on specific target cell populations. Compounds with a specific cytotoxic action are most frequently prepared with monoclonal antibodies as protein vectors (immunotoxins). However, hormones, growth factors, lectins have also been used
25 as vectors for the treatment of cancer.

- The protein that has been most widely used so far for the construction of immunotoxins is ricin chain A; however, several RIPs of type 1 (gelonin, PAP, saporin, momordin, bryodin, barley RIP) have been recently tested in the treatment, e.g., of tumours, autoimmune diseases, transplant rejections,
30 parasitoses, etc.

Since tumour cells are often toxin-resistant and all toxins used so far in therapeutic treatments induce an immune response in treated patients, the identification and purification to homogeneity of new RIPs are of great importance in all therapeutic applications and in particular in the generation of new immunotoxins.

Summary of the invention

The present invention relates to a new RIP protein SEQ ID No: 3, able to inhibit protein synthesis by inactivating ribosomes. Said RIP protein is herein referred to as clavin.

The present invention also concerns the nucleotide sequence SEQ ID No: 2, responsible of the expression of the new RIP protein clavin.

The present invention further includes the conjugates of the aforesaid protein with monoclonal antibodies, hormones, liposomes, growth factors, cytokines, transferrin and peptides, consisting of fragments of said proteins, obtainable by chemical conjugation or by genic recombination techniques whenever applicable.

It is another object of the present invention the Mgr6-clavin conjugate having amino acid composition SEQ ID No: 5, as well as the nucleotide sequence expressing it, corresponding to SEQ ID No: 4.

Detailed description of the invention

SEQ ID No: 1 reports the complete cDNA sequence (i.e., including non-coding 3' and 5' sequences) of clavin, in which:

1-279 = 5' UTR (5' fragment, untranslated);

280-360 = sequence encoding for hypothetical secretion sequence;

361-813 = sequence encoding for mature protein (clavin);

814-1011 = 3' UTR (3' fragment, untranslated) + polyA.

SEQ ID No: 2 refers to the cDNA sequence encoding for mature clavin.

SEQ ID No: 3 corresponds to clavin protein sequence.

SEQ ID No: 4 describes the nucleotide sequence encoding for the Mgr6-clavin immunotoxin produced in pRSET, in which:

1-108 = pRSET sequence containing the 6 histidines and the cleavage site for enterokinase;

109-861 = sequence encoding for ScFv (single Fv) of Mgr6

(109-462 = variable sequence of heavy chain, Bankit I.D. (Gene Bank) 54241, access No. U 61494)

(463-507 = linker sequence)

5 (508-861 = variable sequence of light chain, Bankit I.D. (Gene Bank) 54263, access No. U 61495)

(862-1311 = sequence encoding for clavin).

SEQ ID IN No: 5 corresponds to the protein sequence of Mgr6-claim immunotoxin produced in pRSET

10 The purified protein is >95% pure as shown by SDS/PAGE analysis, N-terminal sequencing and reversed-phase HPLC. The protein molecular weight is approx. 17kDa.

The following examples are conveyed for a better understanding of the protein purification process according to the invention.

15 **cDNA isolation and sequencing**

Total RNA was extracted from *Aspergillus clavatus* IFO 8605 (Institute of Fermentation, Osaka). mRNA was purified using the kits for total RNA and, respectively, mRNA purification (Clontech). The two following primers were synthesized:

20 3' α -primer: 5'-ACGTAAGCTTCTAATGAGAGCAGAGCTT-3' (SEQ ID No: 6)

5' α -primer: 5'-ACGTCTGCAGTGACCTGGACCTGCTTGAACG-3' (SEQ ID No: 7)

Primers were drawn on the basis of the known α -sarcin sequence by assuming a high amino acid sequence homology with the toxin of *Aspergillus clavatus*.

The synthesis of cDNA with 5 μ g mRNA and 3' α -primer was carried out using an
25 appropriate synthesis kit (BRL). Part of the product obtained was added to a reaction mixture for PCR containing Taq polymerase (USB) and the two aforesaid primers; cDNA amplification for a total of 30 cycles was performed using a thermal cyclizer for DNA (Perkin-Elmer).

Each cycle consisted of 1-min denaturation at 94°C, 1-min annealing at 42°C and
30 2-min extension at 72°C; in the final cycle extension at 72°C lasted 8 min.

Amplified cDNA corresponded to the cDNA of clavin, but contained the sequences imposed by 3'- and 5'-primers. Isolation of complete native cDNA of clavin was carried out by the RACE method.

On 3' end, RACE was performed according to Frohman, using the following primer:

5'-GACTCGAGTCGACATCGA(T)₁₇-3' (SEQ ID No: 8), and the adjustment primer: 5'-GACTCGAGTCGACATCG-3' (SEQ ID No: 9).

Primer 5'-ACGTGGATCCTCTACAACCAGAAC-3' (SEQ ID No: 10), which refers to the codons for amino acids 23-29 of mature protein and bearing a restriction site BamHI, was used as a gene-specific primer.

On 5' end, RACE was performed using the 5'-AmpliFINDER RACE Kit (Clontech), and primers

5'-TGAACCAGTGAGGATAG-3' (SEQ ID No: 11)

5'-ACGTCTGCAGGCGCTTGTTCTCATA-3' (SEQ ID No: 12)

referring to the codons for amino acids 47-53 and 18-23 of mature protein were used as gene-specific primers; the latter primer also contains a restriction site PstI.

The various PCR products were purified, digested and subcloned in pUC19. Sequences were analysed using PC GENE software (Intelligenetics).

The complete cDNA sequence obtained is shown in Fig. 1. Said sequence contains an ORF encoding for a 177 amino acid polypeptide chain. The first 27 amino acids represent a signal peptide involved in secretion, while mature protein consists of the 150 amino acids shown in the figure.

Recombinant clavin heterologous expression

Vector pEZZ18 (Pharmacia) was used for recombinant clavin heterologous expression. Said vector directs the expression of fused proteins with a linking synthetic domain IgG (ZZ) based on staphylococcus protein A (Nilsson *et al.*, 1987). Clavin cDNA obtained by PCR with primers based on α -sarcin, as previously described, was re-amplified with 3' primer:

5'-GATCCTGCAGCGACCTGGACTTGCATGAACGAGCAGAAGAACCCAAAG-ACC-3' (SEQ ID No: 13)

and with 3' primer: 5'-ACGTAAGCTTCTAATGAGAGCAGAGCTT-3' (SEQ ID No: 14)

to obtain the mature clavin native sequence, and cloned at restriction sites PstI-HindIII of vector pEZZ18. To obtain pMRS116, fragment EcoRI-PstI was replaced
5 by linker B, which contains a sequence encoding for the cleavage site of factor Xa Ile-Glu-Gly-Arg, in addition to a residue Thr, inserted to preserve restriction site PstI. Linker B was obtained by annealing of the two oligonucleotides:

α -28: 5'-AATTCGATCGAAGGTCGTA CTGCA-3' (SEQ ID No: 15)

α -29: 5'-GTACGACCTTCGATCG-3' (SEQ ID No: 16).

10 For clavin production, construction pMRS116 was propagated in *Escherichia coli* HB 101 [supE44, hsdS20($r_B^-m_B^-$)recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1] and cultured according to the producer's directions (Pharmacia).

Recombinant clavin purification

Culture supernatants were brought to pH 7.6 and added with 1 mM
15 phenylmethylsulfonyl fluoride. Supernatants were injected into an IgG Sepharose Fast Flow column (Pharmacia) equalized with buffer (50 mM Tris/HCl, 150 mM NaCl and 0.05% Tween-20, pH 7.6). The column was first washed with said buffer and then with 5 mM ammonium acetate, pH 5.1; the fusion protein was eluted with 0.5 M ammonium acetate, pH 3.4, freeze-dried, dissolved to 2-8 mg/ml in 20 mM
20 Tris/HCl, 100 mM NaCl, 1 mM CaCl₂, pH 8.0 and digested with Xa factor (Boehringer) at 23°C for 18-24 h in an enzyme/substrate ratio equal to 1:100.

Clavin was purified using a two-phase chromatographic process. In the first phase, the digestion mixture was injected into an S Sepharose Fast Flow column (Pharmacia) equalized with 20 mM sodium phosphate, pH 5.8, and eluted in NaCl
25 gradient; the fusion protein left undigested and clavin were eluted in a single peak. The second phase was still performed on IgG Sepharose Fast Flow column: clavin was collected with the eluent, while the non-digested protein was eluted from the column with 0.5 M ammonium acetate, pH 3.4.

Immunotoxin synthesis and purification (chemical conjugate)

Clavin was derivatized with 40-molar excess ethyl S-acetyl-3-propionthioimide at 4°C for 1 h to obtain an average molar ratio of ethyl S-acetyl-3-propionthioimide groups to toxin molecule equal to 1.2.

- 5 Monoclonal antibody Mgr6, directed against the extracellular domain of ErbB2 and produced from hybridoma Mgr6-C4 MCB c # 762, deposited in the Interlab Cell Line Collection bank (CBA), an international deposit authority, was purified from ascitic liquid as described in Centis *et al.*, 1992. It was then derivatized with 10-molar excess 2-iminothiolane in ethanol at room temperature for 30 min, added
10 with 5,5'-dithio-bis(2-nitrobenzoic acid) to block the free -SH groups and obtain a groups/toxin molecule molar ratio equal to 1:6. The mixture was applied to a BioGel P-6DG column to remove all reagents.

- Derivatized products were mixed using a 5-molar excess clavin, concentrated to a final volume of 1 ml, and added with 100 ul of 0.5 M hydroxylamine, 12.5 mM
15 EDTA, pH 7.2. The solution was stirred at 22°C for 14 h and at 4°C for additional 18 h. The reaction was interrupted by addition of 20 ul of 200 mM N-ethylmaleimide.

The immunoconjugate was purified to homogeneity by ion exchange chromatography.

20 **Recombinant immunotoxin Mgr6-clavin**

Genic construction

- The gene encoding for variable regions of monoclonal antibody Mgr6 was obtained by the "Recombinant Phase Antibody System" kit (Pharmacia) from mRNA of the antibody-producing hybridoma. Said procedure allows the
25 obtainment of DNA encoding for ScFv (Single chain Fv), in which the sequences for the variable regions of heavy and light chains are joined by a linker sequence. The DNA for ScFv was then linked to clavin DNA to obtain the gene for immunotoxin, cloned in commercial vector pRSET (Introvigen). Figs. 4 and 5 show the nucleotide and, respectively, the amino acid sequence of the immunotoxin
30 inserted in pRSET.

The resulting plasmid has the following characteristics:

- a) the fusion protein gene is under the control of the T7 polymerase control;
- b) the resulting protein has at its N-terminal site an extension containing 6 histidines, usable for the purification with IMAC (immobilized metal affinity chromatography) and a cleavage site for enterokinase K.

5 **Recombinant immunotoxin expression in E. coli**

Competent cells B834(DE3)pLys were transformed, plated on 10 LB agar plates containing ampicillin (100 µg/ml), and incubated at 37°C overnight.

Colonies were recovered in 500 ml LB culture medium containing ampicillin (100 µg/ml), glucose (0.5%) and MgSO₄ (1,62 mM), cultured at 37°C under stirring up
10 to OD₆₀₀ = 2.3/2.5, and, after addition of 1 l culture medium (LB, ampicillin, glucose, MgSO₄), amplified at 37°C under stirring up to OD₆₀₀ = 1.2.

Cells were centrifuged and resuspended in 1.5 l culture medium LB supplemented with ampicillin and induced by addition of IPTG (final 1 mM) under stirring at 37°C for 1.5 h. The cell pellet was recovered by centrifugation.

15 **Recombinant immunotoxin purification**

The pellet from 1.5 l culture medium was resuspended in 150 ml of 50 mM Tris-HCl, pH 8, and frozen. 30 ml aliquots were thawed out, sonicated (3 x 20 sec), and centrifuged at 160,00 rpm at 4°C for 30 min.

The resulting pellet was resuspended in 50 ml STET buffer (50 mM Tris-HCl, pH
20 8.5, 8% saccharose, 5% triton X-100, 50 mM EDTA) and the suspension was sonicated (3 x 45 sec) and centrifuged at 30,000 rpm for 20 min. The described washing procedure was repeated twice and twice again with 50 ml of 50 mM Tris-HCl, pH 8.5, and 100 mM NaCl.

Denaturation

25 The sample was resuspended in 30 ml buffer A (50 mM Tris-HCl, pH 8.0, 6 M Gu-HCl, 5 mM imidazole) and incubated at room temperature for at least 2 h.

Immunotoxin purification by IMAC

The sample was centrifuged at 120,00 rpm for 30 min. The supernatant was analysed by chelated metal affinity chromatography (IMAC) using 20 ml Ni²⁺-filled
30 chelating sepharose FF resin (Pharmacia).

The column was washed with 5 vol water, loaded with 5 vol of 0.1 M NiSO₄, washed with 5 vol water and equalized with 5 vol buffer A.

Once the sample had been injected, the column was washed with 5 vol buffer A.

Adsorbed proteins were eluted in step of pH using the following buffers:

- 5 50 mM Tris-acetate, pH 5.5; 6 M Gu-HCl (Buffer B);
50 mM Tris-acetate, pH 4.0; 6 M Gu-HCl (Buffer C).

The immunotoxin was eluted in buffer C.

Immunotoxin reduction

- 10 The sample obtained from IMAC (in a concentration of 1-2 mg/ml) was brought to pH 8.3 with 1 M Tris base. 2 mM EDTA and final 300 mM DTT were added. The resulting product was incubated at room temperature for 3 h.

Immunotoxin refolding

- 15 The reduced sample was rapidly diluted (1:100) in the refolding buffer (50 mM Tris-HCl, pH 8.3, 0.5 M L-Arg, 2 mM EDTA, 4 mM GSSG, 2 mM DTT) and incubated at 10°C for 60 h.

Immunotoxin concentration and dialysis

The sample (ca. 500 ml) was added with Tween-20 (final 0.005%) and concentrated by ultrafiltration through membrane Amicon YM 10.

- 20 Dialysis was carried out using a 10,000-cut-off membrane vs dialysis buffer (50 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 0.005% Tween-20, 10% glycerol).

The resulting product was centrifuged at 120,00 rpm for 30 min and the supernatant containing the immunotoxin was recovered.

Inhibition of cell protein synthesis (chemical immunoconjugate)

- 25 The capacity of clavin and of immunotoxin Mgr6-clavin for inhibiting the protein synthesis was measured on SKBr3 (ErbB2⁺ cells) and on MeWo (ErbB2⁻ cells) cultured in RPMI 1640 containing 10% FCS. The test was carried out essentially as described by Casalini *et al.*, 1993.

- 30 The immunotoxin, the toxin or the monoclonal antibody were diluted in turn in the culture medium. Cells (1.2×10^6) were incubated at 4°C for 3 h in polypropylene test tubes, in 800 ul culture medium containing the appropriate concentrations of immunotoxin, toxin or monoclonal antibody alone. Control cells were incubated

only with the culture medium. Cells were then centrifuged, resuspended in a fresh culture medium and seeded in triplicate in 96-well plates (3×10^5 cells/well).

After incubation at 37°C for 48 h, the culture medium was removed and a fresh culture medium containing [^3H] proline (1 μCi /well) was added. 48 h later, cells
5 were washed and the amount of [^3H] proline incorporated was determined.

In various tests, clavin shows a dose/response effect with IC₅₀ values ranging from 0.1 to 1 μM .

The cytotoxicity of the Mgr6-clavin conjugate is similar to that of ricin A bound to the same monoclonal antibody.

10 **Inhibition of cell protein synthesis (recombinant immunoconjugate)**

The capacity of recombinant immunotoxin Mgr6-clavin for inhibiting the protein synthesis was measured as already described for the chemical immunoconjugate.

The IC₅₀ of recombinant immunotoxin ranges from 0.1 to 1 μM , whereas antibody Mgr6 does not produce any effect. Therefore, clavin is a promising candidate for

15 immunotoxin production.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: MINISTERO UNIVERSITA' RICERCA SCIENTIFICA E
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- (D) STATE: MI
- (E) COUNTRY: ITALY
- (F) POSTAL CODE (ZIP): 20122
- (G) TELEPHONE: 02-5417991
- (H) TELEFAX: 02-54179920

- (ii) TITLE OF INVENTION: A protein capable of inhibiting ribosomal
activity, its preparation and use as a chemical or
recombinant immunoconjugate, and the cDNA sequence
expressing said protein.

- (iii) NUMBER OF SEQUENCES: 16

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: IT FI96A000155
- (B) FILING DATE: 27-JUN-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1011 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (iii) HYPOTHETICAL: NO

- (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Aspergillus clavatus*
- (B) STRAIN: IFO8605

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AACCAGAAAC AAAGGATATG TGGTGAGATT TGTGAGAAAC CAGAACGCTT GGAAAAGAAA	60
ACAAAAGAGA GAAAAGTAAT CACCATCGAT GAGGATATTG TCTGACTCAG AGATCCAACG	120
AAATAATAGT CAACTTCGGA ATGCTTCAAG TCGCCACAT CGAGCTGGGT CAATGGAGTC	180
TCTCGAGTCA GCCAGAGCAC ATATAAAAGC TGCTAGATCC TCGCGGTTCT CCCAGGAAAA	240
CCCAAGATCG TGATCTCAAG CATCTTAACC ACATCCAAAA TGGTCGCAAT CAAGAACCTC	300
GTCCTGGTGG CCCTCACGGC CGTGACCGCC CTTGCGATGC CTTGCGCTCT CGAGGAGCGC	360
GCGGCGACCT GGAATTGCAT GAACGAGCAG AAGAACCCAA AGACCAACAA GTATGAGAAC	420
AAGCGCCTCC TCTACAACCA GAACAATGCC GAGAGCAACG CCCACCACGC GCCTCTCTCC	480
GACGGCAAGA CCGGTAGCAG CTATCCTCAC TGGTTCACCA ACGGCTACGA CGGCGATGGA	540
AAGATCCTCA AGGGCCGCAC GCCCATCAAG TGGGGAAATT CGGACTGCGA CCGCCCTCCC	600
AAGCACAGCA AGAATGGTGA TGGCAAGAAT GACCATTACC TGCTGGAGTT CCCAACATTC	660
CCCGATGGAC ACCAGTATAA TTTCGACTCG AAGAAGCCCA AGGAGGACCC CGGCCCCGCA	720
CGGGTCATCT ACACCTATCC TAACAAGGTG TTCTGCGGCA TTGTTGCCCA CACGAGGGAG	780
AACCAGGGTG ACCTGAAGCT CTGCTCTCAT TAAATGGGCT TGCACAGGGA TATAGTTTGC	840
CATTGGTCGT TCTTCAACCA CGGCTGATAC TATATCGCAT TGGGAAGTGG GGGAGGGAGC	900
TGAATGTTTC ACATATGTTG GTGCAGAACT TGTTCATGT TATCTAGTCA ATCCAGTCT	960
CTCGCTTTGA TATCTATGCA TATTGCACTT CATTGCAAAA AAAAAAAAAA A	1011

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 453 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Aspergillus clavatus*
- (B) STRAIN: IFO8605

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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GCGGCGACCT GGACTTGCAT GAACGAGCAG AAGAACCCAA AGACCAACAA GTATGAGAAC      60
AAGCGCCTCC TCTACAACCA GAACAATGCC GAGAGCAACG CCCACCACGC GCCTCTCTCC      120
GACGGCAAGA CCGGTAGCAG CTATCCTCAC TGGTTCACCA ACGGCTACGA CGGCGATGGA      180
AAGATCCTCA AGGGCCGCAC GCCCATCAAG TGGGGAAATT CGGACTGCGA CCGCCCTCCC      240
AAGCACAGCA AGAATGGTGA TGGCAAGAAT GACCATTACC TGCTGGAGTT CCCAACATTC      300
CCCGATGGAC ACCAGTATAA TTTCGACTCG AAGAAGCCCA AGGAGGACCC CGGCCCGGCA      360
CGGGTCATCT ACACCTATCC TAACAAGGTG TTCTGCGGCA TTGTTGCCCA CACGAGGGAG      420
AACCAGGGTG ACCTGAAGCT CTGCTCTCAT TAA                                     453

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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 150 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Aspergillus clavatus*
- (B) STRAIN: IFO8605

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```

Ala Ala Thr Trp Thr Cys Met Asn Glu Gln Lys Asn Pro Lys Thr Asn
1           5           10           15
Lys Tyr Glu Asn Lys Arg Leu Leu Tyr Asn Gln Asn Asn Ala Glu Ser
20        25        30
Asn Ala His His Ala Pro Leu Ser Asp Gly Lys Thr Gly Ser Ser Tyr
35        40        45
Pro His Trp Phe Thr Asn Gly Tyr Asp Gly Asp Gly Lys Ile Leu Lys
50        55        60
Gly Arg Thr Pro Ile Lys Trp Gly Asn Ser Asp Cys Asp Arg Pro Pro
65        70        75        80
Lys His Ser Lys Asn Gly Asp Gly Lys Asn Asp His Tyr Leu Leu Glu
85        90        95
Phe Pro Thr Phe Pro Asp Gly His Gln Tyr Asn Phe Asp Ser Lys Lys
100       105       110
Pro Lys Glu Asp Pro Gly Pro Ala Arg Val Ile Tyr Thr Tyr Pro Asn
115       120       125
Lys Val Phe Cys Gly Ile Val Ala His Thr Arg Glu Asn Gln Gly Asp
130       135       140
Leu Lys Leu Cys Ser His
145       150

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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1314 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Aspergillus clavatus*
- (B) STRAIN: IFO8605

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

ATGCGGGGTT CTCATCATCA TCATCATCAT GGTATGGCTA GCATGACTGG TGGACAGCAA	60
ATGGGTCGGG ATCTGTACGA CGATGACGAT AAGGATCGAT GGGGATCCCA GGTGCAGCTG	120
CAGGAGTCTG GGGCAGAGCT TGTGAAGCCA GGGGCCTCAG TCAAGTTGTC CTGCACAGCT	180
TCTGGCTTCA ACATTAAAGA CACCTATATG CACTGGGTGA AGCAGAGGCC TGAACAGGGC	240
CTGGAGTGGA TTGGAAGGAT TGATCCTGCG AATGGTAATA CTAAATATGA CCCGAAGTTC	300
CAGGGCAAGG CCACTATAAC AGCAGACACA TCCTCCAACA CAGCCTACCT GCAGCTCAGC	360
AGCCTGACAT CTGAGGACAC TGCCGTCTAT TACTGTGCTA GAGGAGAATA TGATTATCCT	420
TTTCCTTACT GGGGCCAAGG GACCTCGGTC ACCGTCTCCT CAGGTGGAGG CGGTTCAGGC	480
GGAGGTGGCT CTGGCGGTGG CGGATCGTAC ATCGAGCTCA CTCAGTCTCC AGCTTCCTTA	540
GCTGTATCTC TGGGGCAGAG GGCCACCATC TCATGCAGGG CCAGCCAAAG TGTCAGTACA	600
TCTAGGTATA GTTATATGCA CTGGTACCAA CAGAAACCAG GACAGCCACC CAAACTCCTC	660
ATCAAGTATG CATCCAACCT AGAATCTGGG GTCCCTGCCA GGTTCAGTGG CAGTGGGTCT	720
GGGACAGACT TCACCCTCAA CATCCATCCT GTGGAGGAGG AGGATACTGC AACATATTAC	780
TGTCAGCACA GTTGGGAGAT TCCTCGGACG TTCGGTGGAG GGACCAAGCT GGAGCTGAAA	840
CGGGCGGGAT CCCCGBAATT CGCAGCGACC TGGACTTGCA TGAACGAGCA GAAGAACCCA	900
AAGACCAACA AGTATGAGAA CAAGCGCCTC CTCTACAACC AGAACAATGC CGAGAGCAAC	960
GCCCAACACG CGCCTCTCTC CGACGGCAAG ACCGGTAGCA GCTATCCTCA CTGGTTCACC	1020
AACGGCTACG ACGGCGATGG AAAGATCCTC AAGGGCCGCA CGCCCATCAA GTGGGGAAAT	1080
TCGGACTGCG ACCGCCCTCC CAAGCACAGC AAGAATGGTG ATGGCAAGAA TGACCATTAC	1140
CTGCTGGAGT TCCCAACATT CCCCAGTGA CACCAGTATA ATTTGACTC GAAGAAGCCC	1200
AAGGAGGACC CCGGCCCGGC ACGGGTCATC TACACCTATC CTAACAAGGT GTTCTGCGGC	1260
ATTGTTGCCC ACACGAGGGA GAACCAGGGT GACCTGAAGC TCTGCTCTCA TTAG	1314

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 437 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Aspergillus clavatus*
- (B) STRAIN: IFO8605

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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Met Arg Gly Ser His His His His His His Gly Met Ala Ser Met Thr
 1           5           10           15
Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp Asp Asp Lys Asp
 20           25           30
Arg Trp Gly Ser Gln Val Gln Leu Gln Glu Ser Gly Ala Glu Leu Val
 35           40           45
Lys Pro Gly Ala Ser Val Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn
 50           55           60
Ile Lys Asp Thr Tyr Met His Trp Val Lys Gln Arg Pro Glu Gln Gly
 65           70           75           80
Leu Glu Trp Ile Gly Arg Ile Asp Pro Ala Asn Gly Asn Thr Lys Tyr
 85           90           95
Asp Pro Lys Phe Gln Gly Lys Ala Thr Ile Thr Ala Asp Thr Ser Ser
100           105           110
Asn Thr Ala Tyr Leu Gln Leu Ser Leu Thr Ser Glu Asp Thr Ala
115           120           125
Val Tyr Tyr Cys Ala Arg Gly Glu Tyr Asp Tyr Pro Phe Pro Tyr Trp
130           135           140
Gly Gln Gly Thr Ser Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly
145           150           155           160
Gly Gly Gly Ser Gly Gly Gly Gly Ser Tyr Ile Glu Leu Thr Gln Ser
165           170           175
Pro Ala Ser Leu Ala Val Ser Leu Gly Gln Arg Ala Thr Ile Ser Cys
180           185           190
Arg Ala Ser Gln Ser Val Ser Thr Ser Arg Tyr Ser Tyr Met His Trp
195           200           205
Tyr Gln Gln Lys Pro Gly Gln Pro Pro Lys Leu Leu Ile Lys Tyr Ala
210           215           220
Ser Asn Leu Glu Ser Gly Val Pro Ala Arg Phe Ser Gly Ser Gly Ser
225           230           235           240
Gly Thr Asp Phe Thr Leu Asn Ile His Pro Val Glu Glu Glu Asp Thr
245           250           255

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Ala Thr Tyr Tyr Cys Gln His Ser Trp Glu Ile Pro Arg Thr Phe Gly
 260 265 270
 Gly Gly Thr Lys Leu Glu Leu Lys Arg Ala Gly Ser Pro Glu Phe Ala
 275 280 285
 Ala Thr Trp Thr Cys Met Asn Glu Gln Lys Asn Pro Lys Thr Asn Lys
 290 295 300
 Tyr Glu Asn Lys Arg Leu Leu Tyr Asn Gln Asn Asn Ala Glu Ser Asn
 305 310 315 320
 Ala His His Ala Pro Leu Ser Asp Gly Lys Thr Gly Ser Ser Tyr Pro
 325 330 335
 His Trp Phe Thr Asn Gly Tyr Asp Gly Asp Gly Lys Ile Leu Lys Gly
 340 345 350
 Arg Thr Pro Ile Lys Trp Gly Asn Ser Asp Cys Asp Arg Pro Pro Lys
 355 360 365
 His Ser Lys Asn Gly Asp Gly Lys Asn Asp His Tyr Leu Leu Glu Phe
 370 375 380
 Pro Thr Phe Pro Asp Gly His Gln Tyr Asn Phe Asp Ser Lys Lys Pro
 385 390 395 400
 Lys Glu Asp Pro Gly Pro Ala Arg Val Ile Tyr Thr Tyr Pro Asn Lys
 405 410 415
 Val Phe Cys Gly Ile Val Ala His Thr Arg Glu Asn Gln Gly Asp Leu
 420 425 430
 Lys Leu Cys Ser His
 435

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ACGTAAGCTT CTAATGAGAG CAGAGCTT

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 31 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

ACGTCTGCAG TGACCTGGAC CTGCTTGAAC G

31

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GACTCGAGTC GACATCGATT TTTTTTTTTT TTTT

35

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9: ¹⁷

GACTCGAGTC GACATCG

17

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 24 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ACGTGGATCC TCTACAACCA GAAC

24

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TGAACCAAGTG AGGATAG

17

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

ACGTCTGCAG GCGCTTGTTT TCATA

25

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 51 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GATCCTGCAG CGACCTGGAC TTGCATGAAC GAGCAGAAGA ACCCAAAGAC C

51

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ACGTAAGCTT CTAATGAGAG CAGAGCTT

28

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

AATTCGATCG AAGGTCGTAC TGCA

24

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GTACGACCTT CGATCG

16

Claims

- 1 1. Nucleotide sequence SEQ ID No: 2.
- 1 2. Protein capable of inactivating the ribosomal activity, the amino acid sequence
2 SEQ ID No: 3.
- 1 3. Nucleotide sequence SEQ ID No: 4, encoding for the Mgr6-clavin
2 immunotoxin.
- 1 4. Protein sequence SEQ ID No: 5 of Mgr6-clavin immunotoxin.
- 1 5. Conjugates, obtained by chemical conjugation or by genic recombination, of the
2 protein as claimed in claim 2 with hormones, liposomes, monoclonal antibodies,
3 growth factors, cytokines, transferrin and peptides consisting of fragments of said
4 proteins.
- 1 6. The conjugates as claimed in claim 5, wherein the protein is conjugated with
2 monoclonal antibodies.
- 1 7. The conjugates as claimed in claim 5, wherein the monoclonal antibody is
2 Mgr6.
- 1 8. Pharmaceutical compositions containing, as active ingredient, the protein as
2 claimed in claim 2 and/or the conjugates as claimed in any of claims 5 to 7,
3 combined with suitable additives.
- 1 9. Use of the protein as claimed in claim 2 and/or of the conjugates as claimed in
2 any of claims 5 to 7, or mixtures thereof, for the preparation of pharmaceutical
3 preparations useful as anticancer and/or antiviral agents. A protein capable of
4 inhibiting ribosomal activity, its preparation and use as a chemical or recombinant
5 immunoconjugate, and the cDNA sequence expressing said protein.

INTERNATIONAL SEARCH REPORT

Interns al Application No

PCT/EP 97/03359

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K14/38 C07K19/00 C12N15/62 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL/GENEBANK/DBJ databases Accession number U19383, 4 July 1995 Parente et al. "Clavin, type 1 ribosome-inactivating protein from Aspergillus clavatus IF8605" XP002047873 see abstract	1,2
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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
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- *O* document referring to an oral disclosure, use, exhibition or other means
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T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

24 November 1997

Date of mailing of the international search report

09-12-1997

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INTERNATIONAL SEARCH REPORT

Intern. Publication No
PCT/EP 97/03359

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	DATABASE WPI Week 9203 27 November 1991 Derwent Publications Ltd., London, GB; AN 92019323 XP002047697 & JP 03 266 986 A (OTSUKA SEIYAKU KOGYO KK) , 27 November 1991 see abstract	1,2
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Y	F. CENTIS ET AL., : "P185 HER2 NEU epitope mapping with murine monoclonal antibodies" HYBRIDOMA, vol. 11, no. 3, 1992, NEW YORK, NY, US, pages 267-276, XP002047692 see the whole document ---	3-9
Y	WO 95 15341 A (CANCER RESEARCH CAMPAIGN TECHNOLOGY) 8 June 1995 see SEQ.ID.N.14 ---	4,5
A	WO 93 11161 A (ENZON INC) 10 June 1993 see Figure 16A ---	4-9
A	EP 0 524 768 A (IMPERIAL CHEMICAL INDUSTRIES PLC) 27 January 1993 see the whole document, specially examples 4 and 7, claims ---	1-9
A	EP 0 489 931 A (TORAY INDUSTRIES, INC.) 17 June 1992 see abstract and column 2, line 35-45. ---	1-9
A	EP 0 350 230 A (RESEARCH DEVELOPMENT FOUNDATION) 10 January 1990 see abstract ---	1-9
A	WO 95 11977 A (BRISTOL-MYERS SQUIBB COMPANY) 4 May 1995 see abstract and page 1, line 10-21 ---	1-9
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INTERNATIONAL SEARCH REPORT

Intern. Application No

PCT/EP 97/03359

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	CHAUDHARY ET AL.: "A rapid method of cloning functional variable-region antibody genes in Escherichia coli as single-chain immunotoxins" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 87, 1990, WASHINGTON DC, US, pages 1066-1070, XP002047693 see the whole document ---	1-9
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INTERNATIONAL SEARCH REPORT

Informal patent family members

Intern. Application No

PCT/97/03359

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WO 9511977 A	04-05-95	US 5597569 A AU 8094594 A CA 2174943 A EP 0725823 A	28-01-97 22-05-95 04-05-95 14-08-96